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The effects of atoxigenic *A. flavus* co-applied with *T. asperellum* on the population dynamics of toxigenic *A. flavus* in the soil

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Abstract

Aspergillus flavus is an important aflatoxigenic fungal specie infecting crops from production to storage. Contamination of foods and their products by these mycotoxins has become a problem worldwide; in Tanzania, related deaths were reported in 2017. This study focused on assessing the effectiveness of atoxigenic *Aspergillus flavus, Trichoderma asperellum* and a co-inoculated atoxigenic *A.flavus* and *T.asperellum* on the population dynamic of toxigenic *A.flavus* in the soil. A pot experiment was set in the screen house where a known concentration of fungal inoculum was added to the sterile soil. *Aspergillus* spp. were isolated from soil samples by dilution plate techniques on Modified Dichloran Rose Bengal (MDRB) agar and sub-cultured on potato dextrose agar (PDA). Identification was made using morphological and microscopic characteristics of culture in PDA media. Total colony forming units (CFU g^{-1} of soil) and incidence percent were derived from colony count data recorded. R-statistical software version 4.0.4 was used to analyse the collected data. There was a significant difference between treatments on reducing the population (CFU/g) of toxigenic *A.flavus* in soil ($p< 2.2\times10^{-16}$). Co-inoculated *T.asperellum* and atoxigenic *A.flavus* significantly reduced the population (CFU g^{-1} of soil) of toxigenic *A.flavus* to 1.4×10^{5} CFU g^{-1} of soil as compared to control treatment 4.5×10^{5} CFU g^{-1} of soil. There was a strong positive correlation ($p<0.001$) between the incidence percent and population of toxigenic *A.flavus* in the soil under different biocontrol treatments. Co-inoculated T. asperellum and atoxigenic A. flavus had the lowest incidence of 36.3% to other treatments. The findings of this study demonstrate that co-inoculated atoxigenic *A.flavus* and *T.asperellum* was effective biocontrol treatment for reducing the population of *Aspergillus flavus* in the soil ecosystem.

Keywords: *Aspergillus flavus; Biological control; Trichoderma asperellum; Toxigenic A.flavus; Atoxigenic A.flavus*

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Introduction

Maize (*Zea mays L.)* is among the most important staple cereal crops grown in Tanzania predominantly by small-scale farmers under rain-fed conditions for food security and poverty reduction. (Lobulu *et al.,* 2019). Unfortunately, maize is susceptible to infection by toxigenic fungal species causing mycotoxins contamination that has a negative impact on

trade, human and animal health. Maize is at risk of fungal contamination when cultivated in soils with high contamination of toxigenic *A.flavus* fungi at the beginning of the growing season (Patchimaporn *et al.,* 2017). *Aspergillus* is adapted to the tropics and preferably survives in diverse environmental and geographical conditions on plants, well-decomposed organic matters and soils at 37 ^{o}C; this is related to its diverse metabolic activity, high capacity to reproduce and competitive nature of *Aspergillus* strain (Taniwaki *et al.,* 2018). Moreover, Abiotic plant stresses and pest damage in the kernel make plants more susceptible to *Aspergillus flavus* colonization, increasing aflatoxin contamination (Benkerroum, 2020). The most important mycotoxins in maize are aflatoxin, fumonisins, ochratoxins, deoxynivalenol, zearalenone and patulin (Sivparsad *et al.,* 2016). Most staple crops in Africa are prone to aflatoxin because of the colonisation of *A. flavus* S strain which produce aflatoxin as a secondary metabolite (Perrone *et al*., 2014). Aflatoxin B1is listed as the most significant due to its carcinogenic potential linked to the deadliest aflatoxicosis outbreaks (International Agency for Research on Cancer, 2002). In that being the concern, Food and Agriculture Organization (FAO) evaluated that 25% of all crops in the world are prone to contamination by mycotoxins (Reddy *et al*., 2010). Due to the negative impact of aflatoxin on health and trade, it is highly regulated through international standards, which forces trade barriers and reduced marketable produce; as a result, African economies lose about 450 million US$ each year (Bbosa *et al.,* 2013). However, most African countries do not strictly follow a systematic investigation of aflatoxins in cereals (Warth *et al*., 2012). Even in a few countries with strict aflatoxin regulations, most subsistence farmers still consume maize that has not passed surveillance (Wu, 2015). Therefore, meeting maximum tolerable limits set in regulatory standards in products destined for human consumption does not guarantee food safety, especially when large quantities of these food products are part of the meal (Boni *et al.,* 2021). In 2016, Tanzania reported the incidence of aflatoxicosis in two regions of Dodoma and Manyara, whereby 68 people were poisoned and 20 killed linking the incident with the consumption of maize, which contains unacceptably high levels of aflatoxin (Kamala *et al.,* 2018). Consequently, Pre-harvest management techniques consist of biological controls that play a vital role by decreasing transforming fungal populations in favour of biocontrol strain and later reducing aflatoxin contamination to the crops in the field. A recommended biocontrol's such as atoxigenic *A.flavus* with a competitive exclusion mode of action proved effective against toxigenic *A.flavus* (Peles *et al.,* 2021). Either, few studies have been conducted on a combination of two or more biocontrol in one treatment (Sylla *et al*., 2015; Xu *et al*., 2011) with contrasting results. Atoxigenic *A.flavus* inhibits the *A.flavus* mycelium population to increase maize protection from aflatoxin contamination in the field (Khan *et al*., 2021). Furthermore, Garcia *et al*. (2012) reported that co-inoculation of the aggressive strain of microorganisms like *pseudomonas*, *bacillus* and *Trichoderma* significantly reduced Preharvest seed infection by *A. flavus.* For instance, an antagonism study of *Trichoderma* spp against an isolate of *A. flavus* shows that metabolites of *Trichoderma* isolate released on the growth medium inhibit colony growth of *A. flavus* isolate and the production of aflatoxin B1 (Ren *et al.,* 2022). In addition, *Trichoderma* protects the plant from attacks of other pathogens (Gajera *et al*., 2015) and enhances plant resilience to drought stress and insect damage, which influence aflatoxin occurrence. Thus, a biocontrol agent with *Trichoderma* has additional benefits in the management of *A.flavus* and the reduction of aflatoxins in maize grains (Caceres *et al*., 2020). This study aimed to determine the effects of atoxigenic *A. flavus* co-applied with *T. asperellum* on the population dynamics of toxigenic *A. flavus* in the soil.

Material and methods

Study area and duration

The experiment was conducted from May to July 2022 at the Plant Pathology Laboratories of Sokoine University of Agriculture (SUA), located at latitude 6^{0} 49′ 27″ S, longitude 37^{0} 39′ 48″ E and elevation of 509 meters above sea level.

Experimental design

The *in vitro* experiment was laid as a one-factor experiment in a completely randomized design (CRD) with four replicates for each treatment.

The experimental factor was biocontrol agents in four (4) levels: T0-Toxigenic *A.flavus*, T1-Atoxigenic *A.flavus*, T2-*Trichoderma asperellum*, T3-Co-inoculated atoxigenic *A.flavus* and *T.asperellum*.

Inoculum preparation and soil inoculation

Toxigenic *Aspergillus flavus* and atoxigenic *A.flavus* culture (TGS 55-6) were obtained from the International Institute of Tropical Agriculture (IITA) laboratories - in Dar es Salaam. In the laboratory *A.flavus* inoculum was prepared following the method from Abdelaziz *et al*. (2022); in short *A.flavus* cultures were grown in plates containing potato dextrose agar (PDA). Plates were incubated at 28 ^{0}C for seven days. Conidia were collected and suspended in sterile distilled water. Thereafter, the number of conidia was determined using a hemocytometer (Neubauer, Marienfeld, Germany). The original suspension was diluted to a concentration of 1 x 10 ^{3}. The spores were counted under a light microscope 40x and calculated following the formula (Kamaruzzaman *et al.,* 2016);

$$\text{Total spores/ml} = \frac{\text{No of spores} \times \text{dilution factor}}{\text{Number of smallest squares counted}} \times 4000\ (40x)$$

Commercially available *T. asperellum* (bought from Real IPM, Arusha, Tanzania.); is a registered biocontrol agent for root-knot nematodes and another soil-borne pathogen in crops was used. The inoculum was prepared following the manufacturer's instructions by dissolving 1 ml into 1000 ml of sterile distilled water to obtain a concentration of 1 x 10^{9} CFU ml $^{-1}$. Disinfected 4 kg pots were used, before potting a forest soil was sterilized through steam/heat treatment at 80 ^{0}C for two hours to ensure soil is free from other soil fungal species as described by Li YB *et al.* (2022) protocol. Then soil sample was sent to the plant pathology laboratory where 10 g of soil was diluted in sterile distilled water to make a suspension that was spread in the Modified Dichloran Rose Bengal (MDRB) to test the growth of any fungal species before inoculation. Three days after testing soil was found sterile. During inoculation, the hand sprayers was used to spray the inoculum of *T. asperellum* isolate, toxigenic *A.flavus*, Atoxigenic *A.flavus* and co-inoculation of *T. asperellum* and atoxigenic *A.flavus* in the soil by applying 10 mls of each inoculum singly. Inoculation of treatments was performed following a slightly modified protocol described by Abdelaziz *et al*. (2022); whereby, 20 ml of inoculum per pot was spayed in the soil and then mixed thoroughly to uniform moisture before potting.

Preparation of media

Enumeration media Modified Dichloran Rose Bengal (MDRB) agar was prepared following the manufacturer's instruction by dissolving 32.15 g of DRBM powder into 1000 ml of sterile distilled water, and the suspension was autoclaved at 121 ^{0}C and 15 Pressure per Square Inch (PSI) for 15 minutes. The sterilized media was left for 20 minutes to cool at 45 ^{o}C and poured into 90 mm sterile petri dishes. The petri dish was left to cool at room temperature before inoculating the following day. The growth media potato dextrose agar (PDA) was prepared by dissolving 39 g of PDA powder into 1000 ml of sterile distilled water, and the media were autoclaved at 121 ^{0}C, 15 PSI for 15 minutes. The media was left to cool for 45 ^{0}C at room temperature and then poured into sterile distilled petri plates at laminar flow overnight to solidify before use. In this study, coconut milk agar media (CMA) was used to distinguish toxigenic *A.flavus* from atoxigenic *A.flavus*. Locally available coconut milk (protein 1.8 g, carbohydrate 3.1 g/100 g, total fat 17.8 g, saturated fat 15.7 g) was poured into a sterile jar followed by a 2N solution of NaOH to adjust the pH of the media to 6.8 and then 15 g of agar was added. The mixture was autoclaved (121^{o}C) for 20 minutes, then left to cool until 45^{o}C and poured into sterile Petri dishes (Iram *et al.,* 2018).

Isolation and identification of fungal isolates from soil samples

Soil samples were collected from the pots in the screen house at 7, 14 and 21 days after inoculation. During sample collection, 10 g of a soil sample was obtained from a well-mixed soil by using a hand trowel, soil samples were passed through a 250 µm sieve (No.4), later packed into a paper bag, and finally sent to the plant pathology laboratory for serial dilution to isolate the fungal species (Abdelaziz *et al*., 2022). The dilution method technique was used by suspending 1 gram of soil sample in 9 ml of sterile distilled water. The suspension was homogenized for two minutes and serially diluted up to 10 $^{-4}$ by adding one ml from

preceding diluted suspension into the test tube containing 9 ml sterile distilled water. The aliquot of 100 μl were inoculated aseptically using the micro applicator onto Petri dishes containing Modified Dichloran Rose Bengal (MDRB) in the laminar flow and spread on the surface of culture media. The fungal culture was incubated for five days at 28^0C and removed afterwards for macroscopic identification (Cotty,1994). Petri dishes containing 30 to 60 colonies were used for counting and recording the number of colonies that emerged from the soil samples. From the number of colonies recorded, *A.flavus* strains L and S per gram of soil were calculated and expressed as colony-forming units per gram of soil (CFU g^{-1}) following the formula *(i)* below, while the incidence of fungal species calculated following formula (*ii)* below:

$$\text{CFU/g soil} = \frac{\text{Number of colonies of fungal species}}{\text{Amount plated x Dilution factor}} \quad \ldots i$$

$$\text{Incidence (\%)} = \frac{\text{Number of fungal species isolates}}{\text{Total number of fungal species}} \times 100 \ldots ii$$

Identification

The macroscopic identification of *A.flavus* strains was based on the cultural and morphological features of the colonies (figure 1). Overall, *A.flavus* had a yellow to bright green or olive green colony appearance surrounded by a white circle that were eventually covered by conidia, the texture of the colony was woolly with a floccose center (Fakruddin *et al.,* 2015). The macroscopic identification of *T. asperellum* was based on morphological characteristics (figure 1). White mycelia with cottony texture and dark green spores arranged in five (5) concentric rings of either, dark green or whitish at five days' post-inoculation on PDA media and were identified as *T. asperellum* (Moussa *et al.,* 2023). The center of the Petri dishes containing coconut milk agar was inoculated with *A.flavus* isolates and then incubated in the dark at 28°C. After 7 days of incubation, the colonies were subjected to long-wave ultraviolet (UV) light of 365 nm to check the absence or presence of a blue fluorescence ring surrounding the colonies (figure 1) based on the method described by (Iram *et al.,* 2018).

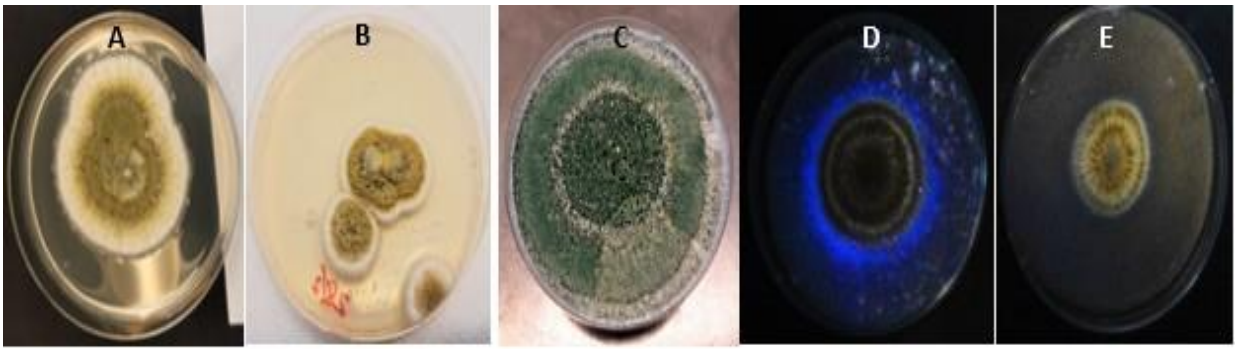

Figure 1.
Colonies of toxigenic A.flavus (A) and atoxigenic A.flavus (B); Colony of Trichoderma asperellum (C);Toxigenic A.flavus (D) and atoxigenic A.flavus (E) under UV light.

Data Analysis

In this study, the R-statistical software version 4.0.4 was used to analyse the collected data for the applied treatments (R Core Team, 2021). The normality of collected data to check if they would meet the assumption of analysis of variance, (ANOVA) was tested using the Shapiro-Wilk test at a 5% significance level. The generalized linear model (GLM) following a Poisson distribution was used to account for the variance error before doing ANOVA at a 5% significance level. The Correlation analysis was done to estimate the statistical relationship between incidence percentage and population (CFUg^{-1}) of toxigenic *A.flavus* in the soil at a 5% significance level.

The statistical model used was;

$$Y = \mu + A + \varepsilon \quad \ldots iii$$

Where: $\boldsymbol{\mu}$ is the grand mean, the letter $\boldsymbol{A}$ is a biocontrol and $\boldsymbol{\varepsilon}$ is a random error term.

Results

Effect of biocontrol treatments on the population (CFU/g) of toxigenic A.flavus in soil

There was a significant difference between treatments on reducing the population (CFU g^{-1}) of toxigenic A.*flavus* in soil ($p<2.2\times10^{-16}$). Generally, the use of three biocontrol treatments significantly reduced the population (CFU g^{-1}) of toxigenic A.*flavus* in the soil as compared to the control treatment. Co-inoculation of T.*asperellum* and atoxigenic A.*flavus* significantly minimized the population (CFU g^{-1}) of toxigenic *A.flavus* to 1.4×10^{5} CFU g^{-1} of soil sample as compared to control treatment 4.5×10^{5} CFU g^{-1} of soil sample) (figure 2).

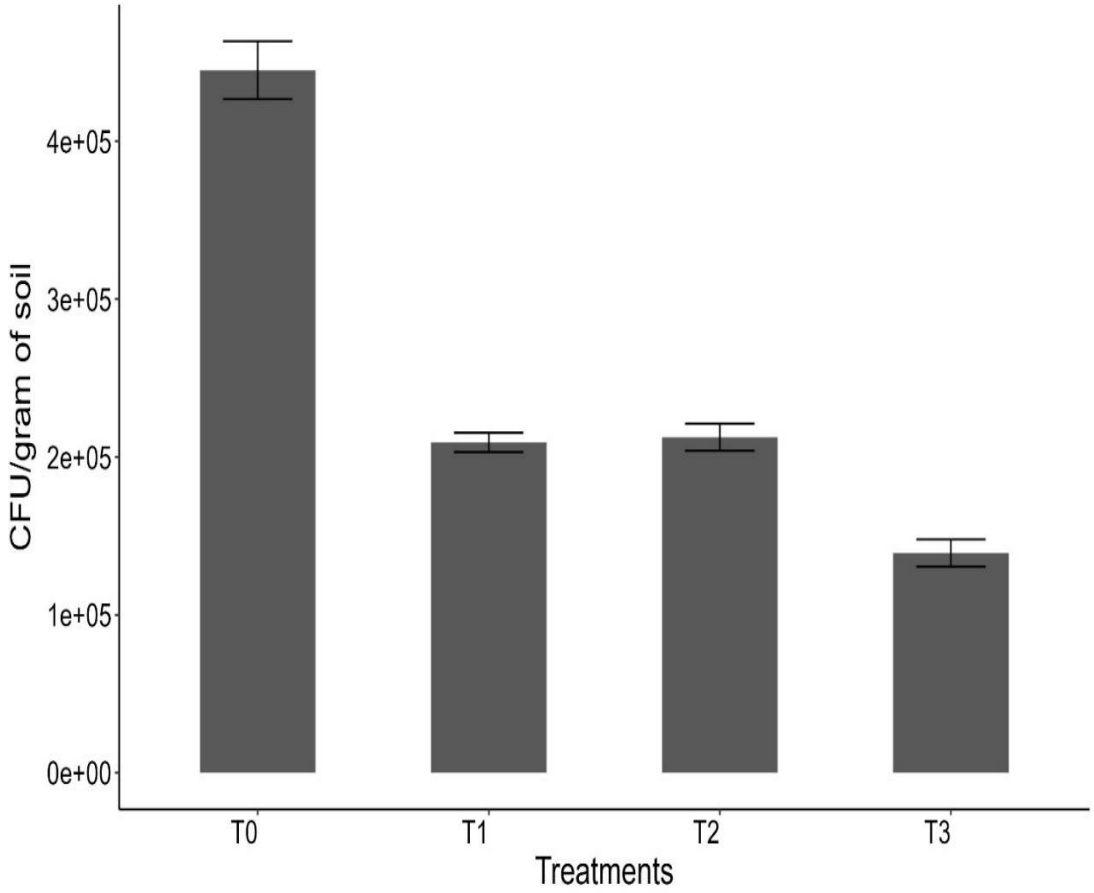

Figure 2.
The population of toxigenic A.flavus in the soil
Key: T0 – Control (Toxigenic A.flavus), T1- Atoxigenic A.flavus, T2- T.asperellum, T3- Co-inoculated atoxigenic A.flavus and T.asperellum.

Moreover, the effect treatments over time were noticed when co-inoculated *T.asperellum* and atoxigenic *A.flavus* significantly affected the population (CFU g^{-1}) of toxigenic *A.flavus* in soil samples (p=0.028). The lowest population (CFU g^{-1}) 1.1×10^{5} of toxigenic *A.flavus* in soil samples was recorded on the 21st day after inoculation compared to 7th and 14th days after co-inoculating the soil with the *T.asperellum* and atoxigenic *A.flavus* (figure 3).

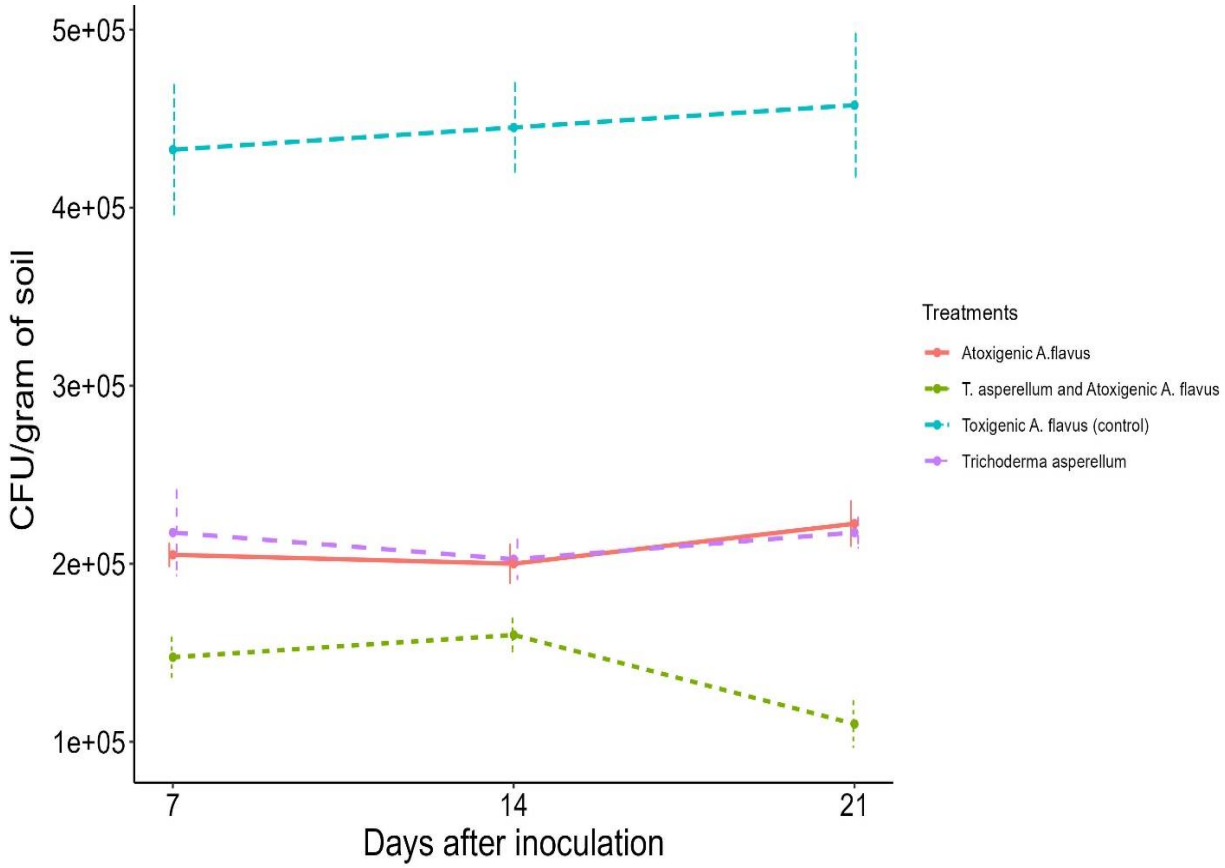

Figure 3.
Population of toxigenic A.flavus over time after application of biocontrol treatments

Effect of biocontrol treatments on incidence percentage of toxigenic A.flavus in soil

A significant difference between the applied treatments on the incidence of toxigenic *A.flavus* in the soil was observed ($p < 2.2 \times 10^{-16}$). Soil co-inoculated with *T.asperellum* and atoxigenic *A.flavus* resulted in a lower incidence of toxigenic *A.flavus* (36.3%) than other treatments (figure 4). However, there was no significant variation in treatments applied over time after inoculation in reducing the incidence (%) of toxigenic *A.flavus* ($p = 0.99$).

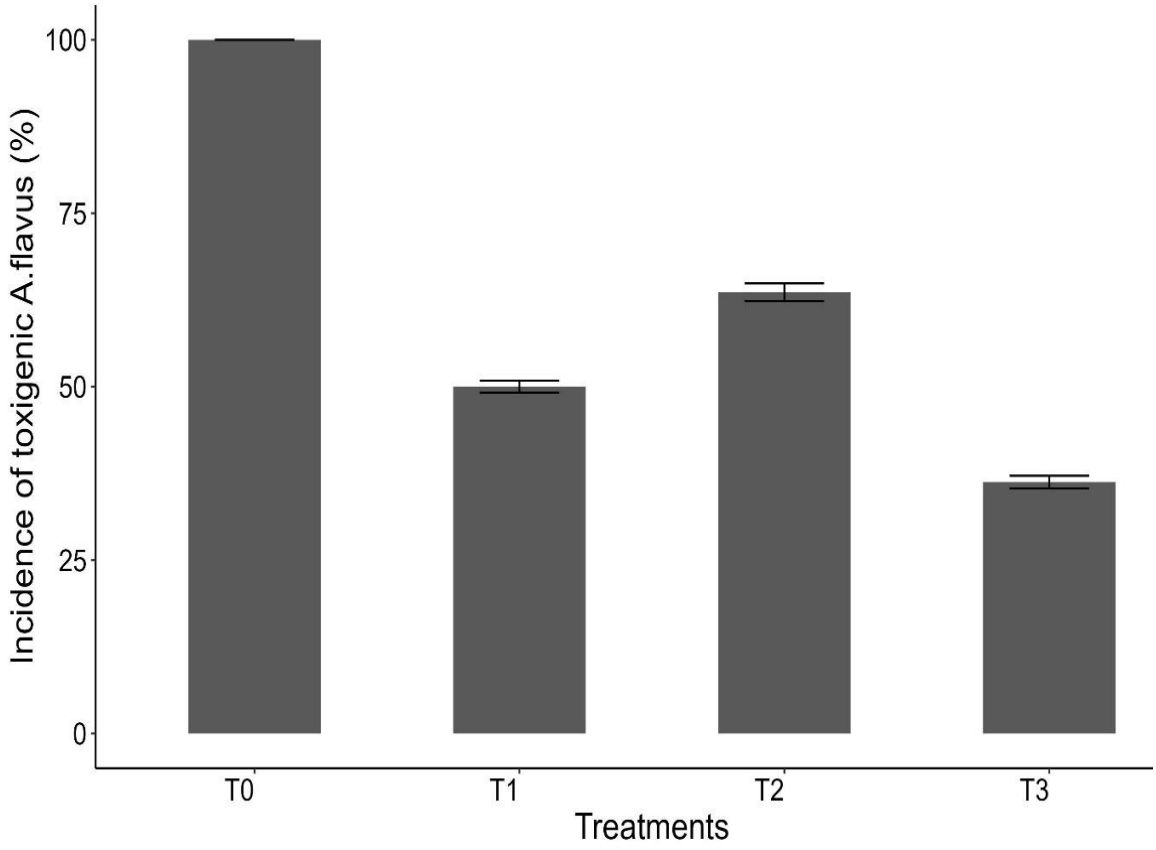

Figure 4.
Incidence percentage of toxigenic A.flavus in the soil

Correlation between population (CFU g^{-1}) and incidence of toxigenic A.flavus in the soil

There was a strong significant positive correlation between population (CFU g^{-1}) and incidence of toxigenic *A.flavus* in the soil (figure 5). The results shows that, as the incidence of toxigenic *A.flavus* were increasing it led to the increase of *A.flavus* population in the soil too.

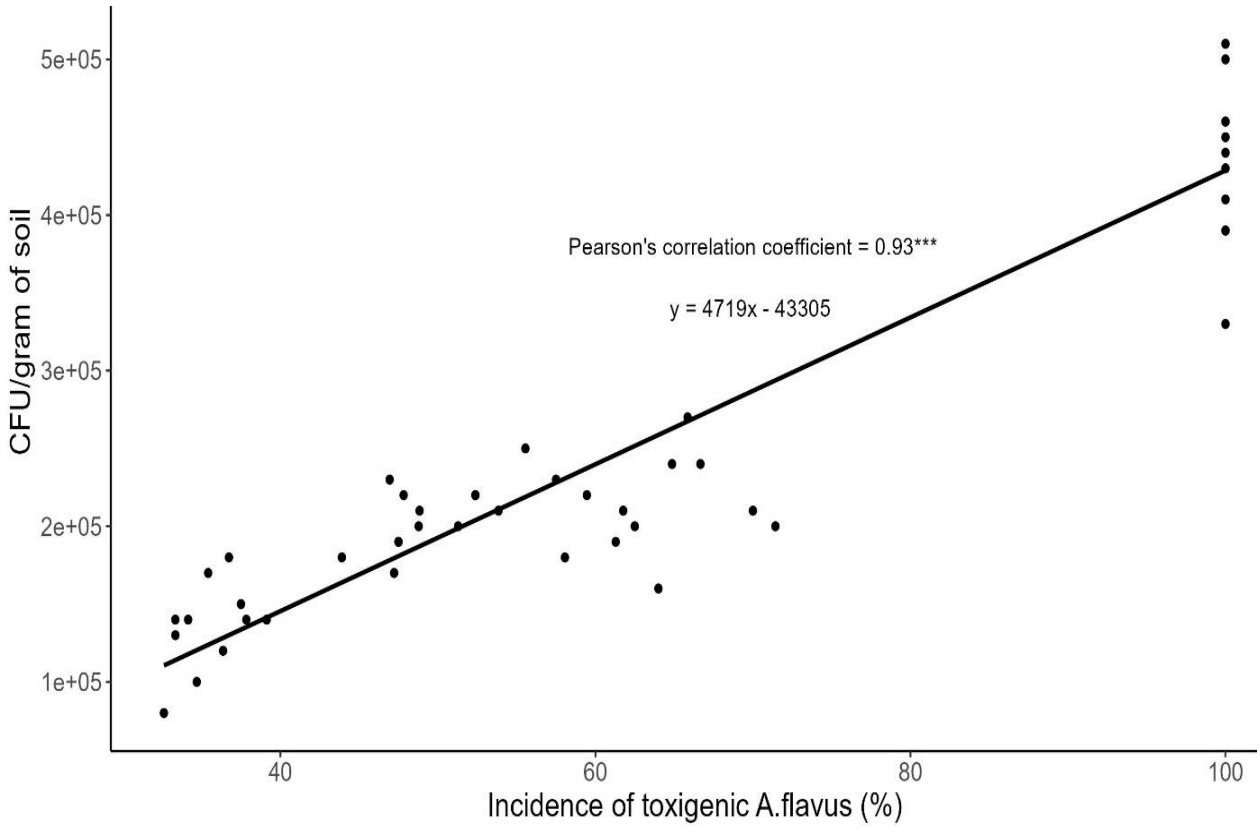

Figure 5.
Pearson correlation between population (CFU g^{-1} of soil) and incidence percentage of toxigenic A.flavus in the soil sample. *** stands for significant at a 0.1% level of probability.

Discussion

Recently, the use of microbial biocontrol agents such as bio fungicides has continuously increased due to public awareness of the risk imposed by pesticide residues in food and its adverse impact on biodiversity. However, most studies related to the biological management of *A.flavus* have focused on individual biocontrol mechanisms rather than a combination of several biocontrol mechanisms that provide multiple effects against pathogenic fungi. This study entails assessing the combined effect of biocontrols to reduce the population of aflatoxigenic fungi in the soil.

Population dynamics of A.flavus

Study findings indicated that the decrease in the population of toxigenic *A.flavus* in the soil media related to the ability of biocontrol strains that support the theory of competitive exclusion through vigorous propagation, antibiosis and fast colonization made by toxigenic *A.flavus* and *T. asperellum*. The effectiveness of biocontrol agents used in reducing the population of *A.flavus* in the soil media are in conformity with findings from Abbas *et al.* (2004) and Zablotowicz *et al.* (2007) which reported that *A.flavus* population in the soil can range from 200 CFU to >300,000 CFU. These findings are also in line with the study by Moreira *et al.* (2021), which reported the potential of combining *Bacillus sp.* and *T. asperellum* against banana plant disease and its effect on the improvement of plant growth and development. The competitive approach of biocontrol may comprise the production of specific metabolites to inhibit further growth of toxigenic *A.flavus*, further affecting its population growth. On the other hand, the *Trichoderma* species' mode of action involves utilizing available resources for toxigenic *A.flavus*, to starve them with none or very little available for their growth and development, and therefore delaying the initial establishment of toxigenic A.flavus (Sivparsad *et al.*, 2016). Another possible mode of action used by Trichoderma in suppressing the growth and later reducing the

population of *A.flavus* might be antibiosis, this too has been reported in previous studies by (Schuster and Schmoll 2010), which revealed the antagonistic ability of Trichoderma against toxigenic *A.flavus*. For effective reduction of the toxigenic *A.flavus* population in the soil, this study shows that a higher population of competitive biocontrols is essential to consider, especially when atoxigenic *A.flavus* is involved. This agrees with the study by Dorner (2010), which reported that for effective competitive exclusion, biocontrol strain must be present at highly competitive levels. Another study by Mfaume *et al.* (2019) shows that an average of 21.4 % population of atoxigenic *A.flavus* successfully managed 79.5 % of toxigenic *A.flavus*.

Incidence of *A.flavus*

The result of this study shows the variation of *A.flavus* incidence under different biocontrol treatments, the higher incidence in a control treatment observed as compared to other treatments, which indicated that, without proper management of toxigenic *A.flavus* higher incidence, will lead to its spreading and later contamination of food crops. Nyongesa *et al.* (2015) made a similar observation, on the correlation between higher incidences of toxigenic *A.flavus* found in the soil, which is the primary inoculum to that found in the maize sample. As indicated in the study by Abbas *et al*. (2004) the incidence *A.flavus* isolates can range from 50 to 80%, therefore biocontrol used has proven to have an implication in reducing the incidence of *A.flavus* to acceptable levels Furthermore, a successful reduction of toxigenic *A.flavus* incidence was observed when the co-inoculated atoxigenic *A.flavus* and *T. asperellum* were used. This might be due to the synergism of the biocontrol agent's mode of action against toxigenic *A.flavus* in the soil media where *T. asperellum* may arrest its further growth. In contrast, the higher incidence of atoxigenic *A.flavus* in the soil may reduce the necessary resources to colonize the soil. These findings are in agreement with the study by Mahuku *et al.* (2022) and Ren *et al*. (2021), which reported that areas with dominant toxigenic *A.flavus* needs timely application of atoxigenic *A.flavus* to increase its frequency in the soil and later reduce toxigenic *A.flavus*. In addition, the fast colonization of *T. asperellum* and its ability to release metabolites arrests the growth of *A.flavus* and therefore stop its further widespread.

Conclusion

This study highlights the potential of a co-inoculated biocontrol agent against toxigenic *A.flavus*. A biocontrol containing a combination of *T. asperellum* and atoxigenic *A.flavus* was more effective in reducing the incidence and population of aflatoxigenic *A.flavus*. Therefore, this co-inoculation bio-control would provide multiple advantages, including reducing the accumulation of aflatoxins in food crops while serving as an eco-friendly biocontrol.

Recommendation

Further studies on the influence of these biocontrol agents in reducing aflatoxin contamination under field conditions are required to evaluate their actual potential as a bio fungicide in an eco-friendly agricultural ecosystem.

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